The mitochondrial ubiquitin ligase plays an anti-apoptotic role in cardiomyocytes by regulating mitochondrial fission

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Abstract

Apoptosis plays a critical role in the development of myocardial infarction. Cardiomyocytes are enriched with mitochondria and excessive mitochondrial fission can trigger cellular apoptosis. Recently, the mitochondrial ubiquitin ligase (MITOL), localized in the mitochondrial outer membrane, was reported to play an important role in the regulation of mitochondrial dynamics and apoptosis. However, the underlying mechanism of its action remains uncertain. The present study was aimed at uncovering the role of MITOL in the regulation of cardiomyocyte apoptosis. Our results showed that MITOL expression was up-regulated in cardiomyocytes in response to apoptotic stimulation. Mitochondrial ubiquitin ligase overexpression blocked dynamin-related protein 1 accumulation in the mitochondria, and attenuated the mitochondrial fission induced by hydrogen peroxide. Conversely, MITOL knockdown sensitized cardiomyocytes to undergo mitochondrial fission, resulting in subsequent apoptosis. These findings suggest that MITOL plays a protective role against apoptosis in cardiomyocytes, and may serve as a potential therapeutic target for apoptosis-related cardiac diseases.

Keywords: MITOL ● apoptosis ● mitochondrial fission ● cardiomyocytes ● doxorubicin ● hydrogen peroxide

Introduction

Apoptosis is essential for normal development and maintenance of tissue homeostasis [1, 2]. In the cardiovascular system, for example, apoptosis participates in shaping the cardiac and vascular structures during early morphogenesis and in regulating the growth of established and differentiated cardiovascular tissues at later developmental stages [2]. However, there is growing body of evidence, which shows that excessive apoptosis is related to many cardiovascular diseases such as myocardial infarction, cardiomyopathy, cardiac hypertrophy and anthracycline-induced cardiotoxicity, *etc.* [3–6]. Reactive oxygen species play an important role in triggering apoptosis [7, 8], but the molecular mechanism by which they exert their effects remains to be fully understood.

Cardiomyocytes are enriched with mitochondria, which play an essential role in various cellular phenomena including ATP synthesis, lipid and iron metabolism, calcium buffering and cell death [9, 10]. It has been recently demonstrated that the mitochondrial morphology is an important determinant of mitochondrial function [2, 11, 12]. Mitochondria constantly undergo fusion and fission, which are

necessary for the maintenance of organelle fidelity [13–15]. At the same time, growing evidence has shown that abnormal mitochondrial fusion and fission also participate in the regulation of apoptosis [10, 11]. Mitochondrial fusion is able to inhibit apoptosis, while mitochondrial fission is involved in the initiation of apoptosis [11, 13, 16]. Although mitochondrial malfunction has been shown to be involved in brain and skeletal muscle disorders [17–20], it remains largely unknown as to whether the abnormal mitochondrial fission and/or fusion play a role in regulating cardiomyocyte survival and death.

The mitochondrial ubiquitin ligase (MITOL), also known as MARCH5/RNF153 was recently reported to play a functional role in mitochondria [21]. It can potentially modulate mitochondrial fission as well as mitochondrial morphology [21, 22]. Mitochondrial ubiquitin ligase is characterized by its four transmembrane domains for the binding of mitochondrial fission proteins namely, human mitochondrial Fission 1 (hFis1), dynamin-related protein 1 (Drp1) and mitofusin 2 [23–25]. However, whether MITOL plays a functional role in the mitochondrial dynamics and apoptosis of cardiomyocytes remains unknown. Accordingly, the current study was aimed at uncovering the role MITOL in the regulation of cardiomyocyte apoptosis induced by hydrogen peroxide (H_2O_2).

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Materials and methods

Cell cultures and treatment

Mouse HL-1 cardiomyocytes, kindly provided by Dr. William C. Claycomb, were cultured in Claycomb media supplemented with 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mol/l norepinephrine (Sigma-Aldrich), 2 mmol/l L-glutamine (Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Invitrogen) in a humidified 5% CO_2 incubator at 37°C [26]. Primary rat cardiac myocytes (Lonza, Walkersville, MD, USA) were cultured in Rat Cardiac Myocyte Growth Media (RCGM; Lonza) containing horse serum, foetal bovine serum and gentamicin/amphotericin-B, further supplemented with 200 μM 5-bromo-2′-deoxyuridine in a humidified 5% CO_2 incubator at 37°C. Cardiomyocytes were treated with identical concentration of H_2O_2 plus ferrous sulphate for 1 hr and further cultured in normal culture medium without H_2O_2 and ferrous sulphate as we have previously described [27].

Construction of MITOL expression vector

Myc-DDK-tagged ORF clone of Mus musculus membrane-associated ring finger (C3H4)5 (MITOL) cDNA was cloned into pCMV6-Entry (Origene, Rockville, MD, USA) according to the manufacturer's instruction. β-galactosidase (β-gal) was used as a control.

Construction of MITOL RNA interference (RNAi) vectors

For HL-1 cells, the MITOL RNAi sense sequence was 5′-AGGAGCATT-TAAGGTTTACTTCAA ACAGC-3′, and the antisense sequence was 5′-GCTGTTTGAAGTAAACCTTAAATG CTCCT-3′. The scramble MITOL RNAi sense sequence was 5′-GCACTACCAGAGCTAACTCAGATAGTACT-3′, and the antisense sequence was 5′-AGTACTATCTGAGTTAGCTCTGGTAGTGC-3′. They were cloned into the pGFP-V-RS shRNA retroviral vector (Origene) according to the manufacturer's instructions.

For primary neonatal rat cardiomyocyte, the MITOL-shRNA sense sequence was 5′-CTAAGTGGGTTCACCAGGCTTGTCTACAA-3′, and the antisense sequence was 5′-TTGTAGACAAGCCTGGTGAACCCACTTAG-3′. The scramble MITOL-shRNA sense sequence was 5′-GCACTACCAGAGCTAACT-CAGATAGTACT-3′, and the antisense sequence was 5′-AGTACTATCTGAGT-TAGCTCTGGTAGTGC-3′. They were cloned into the pGFP-C-shRNA lentiviral vector (Origene) according to the manufacturer's instructions.

Plasmid transfection

Using Lipofectamine 2000 (Invitrogen), we transfected the cells with plasmids expressing MITOL, MITOL-siRNA, MITOL-shRNA vectors or empty/scrambled siRNA/shRNA vectors according to the manufacturer's instructions.

Preparation of mitochondrial fractions

Mitochondrial fractions were prepared as we described [28]. Briefly, cells were washed twice with PBS and the pellet was suspended in 0.2 ml of buffer A [20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Ethylene Glycol Tetraacetic Acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.1 mM PhenylMethane Sulfonyl Fluoride (PMSF), 250 mM sucrose] containing a protease inhibitor cocktail. The cells were homogenized by 12 strokes in a Dounce homogenizer. The homogenates were centrifuged twice at 750 × g for 5 min. at 4°C. The supernatants were centrifuged at 10,000 × g for 15 min. at 4°C to collect mitochondria-enriched heavy membranes.

Analysis of mitochondrial fission

Mitochondrial fission was analysed by staining mitochondria as we and others have described earlier with some modification [29, 30]. Briefly, cells were plated onto the coverslips coated with 0.01% poly-L-lysine. After treatment, they were stained for 20 min. with 0.02 μM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA). Mitochondria were imaged using a laser scanning confocal microscope (Zeiss LSM710 META, Dublin, CA, USA). To quantitatively analyse cells with mitochondria fission, those cells with disintegrated mitochondria were taken as mitochondrial fission. The percentage of cells with fragmented mitochondria relative to the total number of cells was presented as the mean ± S.E.M. of at least three independent experiments, counted by an observer blinded to the experimental conditions. A range of 100–150 cells in 20–30 random fields were counted.

Immunoblot analysis

Immunoblotting was carried out as previously described [31]. Cells were lysed for 1 hr at 4°C in a lysis buffer (20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM DTT, 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) containing a protease inhibitor cocktail. Samples were subjected to 12% SDS-PAGE and transfected to PVDF membrane (Millipore, Billerica, MA, USA). Equal protein loading was controlled by Ponceau Red staining of membranes. Blots were probed using primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Anti-MITOL polyclonal antibody was from Lifespan Biosciences. Anti-cleaved-PARP, anti-Actin, anti-β-tubulin and anti-Drp1 antibody were from Cell Signalling Technology Inc (Danvers, MA, USA). Anti-Bid, -bad - caspase-3 antibodies were from Santa Cruz Biotechnology Inc (Dallas, TX, USA). Antigen-antibody complexes were visualized by enhanced chemiluminescence. The protein band intensity was quantified by ImageJ (National Institutes of Health, Bethesda, MD, USA) using protocol written by Luke Miller, November 2010 (http://www.lukemiller.org/ImageJ_gel_analysis.pdf). Briefly, the density of each sample was first quantified with image J, then the percent value of each sample and that of standard was calculated. Finally, the relative density was calculated by dividing the percent value of each sample by the percent value of each standard.

DNA fragmentation and apoptosis assays

DNA fragmentation was monitored using the cell death detection ELISA kit (Roche, Branford, CT, USA) as we have described
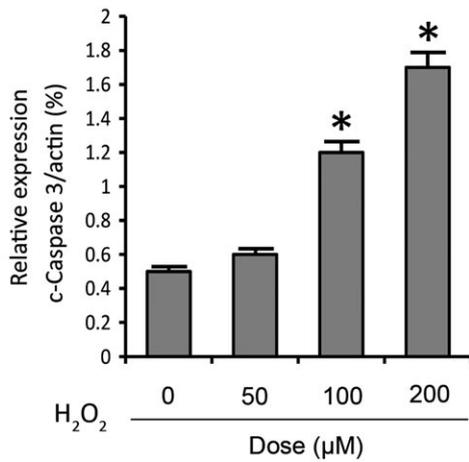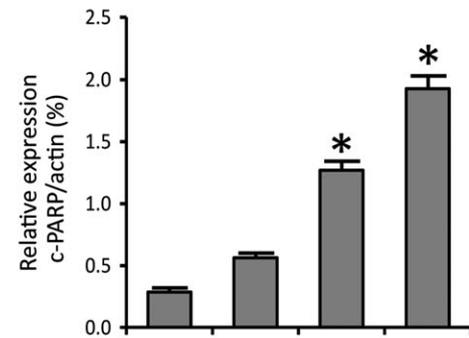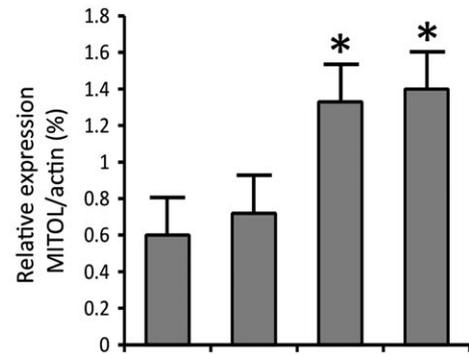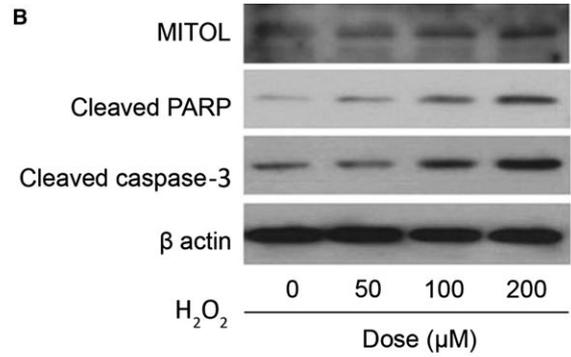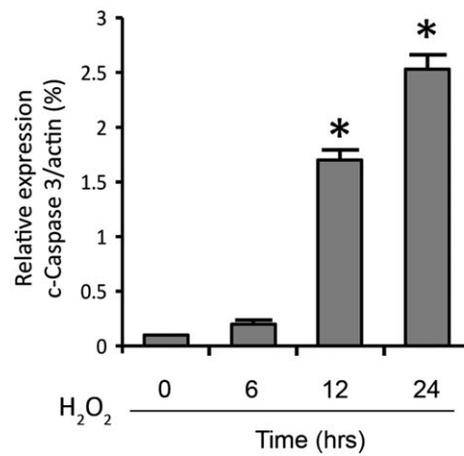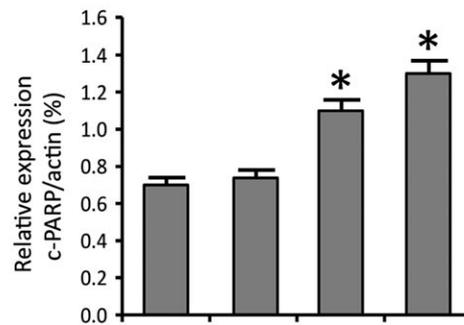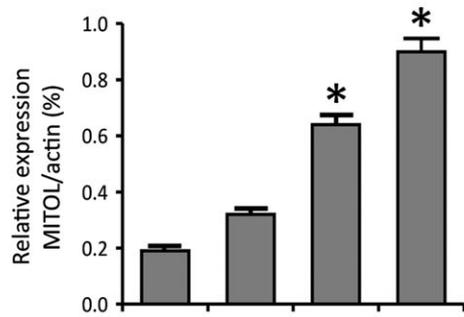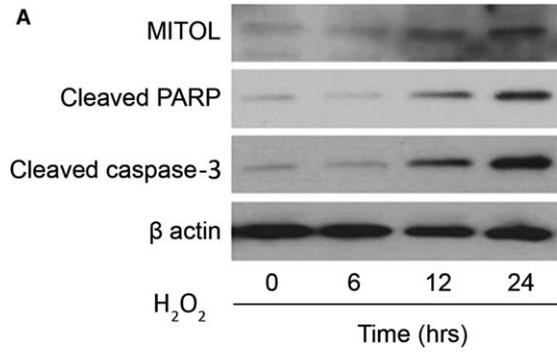
Fig. 1 Hydrogen peroxide exposure leads to a time- and dose-dependent up-regulation of MITOL. (**A**) HL-1 cells were stimulated with 200 μM hydrogen peroxide (H_2O_2) and then harvested at the indicated time for immunoblotting. (**B**) HL-1 cells were stimulated with the indicated doses of H_2O_2 and then harvested for immunoblotting. (**A** and **B**) Immunoblots showing MITOL expression, PARP cleavage and caspase-3 cleavage upon treatment of cells with H_2O_2. β-actin served as a loading control. The densitometry data were expressed as the mean ± S.E.M. of three independent experiments. The relative expression level of protein was determined by dividing the percent value of specific protein to that of standard. *$P < 0.05$ *versus* non-treated control.

elsewhere [28]. Briefly, the anti-histone monoclonal antibody was added to the 96 well ELISA plates and incubated overnight at 4°C. After recoating and three rinses, the cytoplasmic fractions were added and incubated for 90 min. at room temperature. After three washes, bound nucleosomes were detected by the addition of anti-DNA peroxidase monoclonal antibody and reacted for 90 min. at room temperature. After the addition of the substrate, the optical density was determined at 405 nm using an ELISA reader. For apoptosis analysis, a terminal deoxynucleotidyl transferase-mediated Dutp nick-end-labelling (TUNEL) kit (Clontech, Mountain View, CA, USA) was used according to the kit's instructions. One hundred and fifty to two hundred cells were counted in 20–30 random fields in each group.

## Detection of caspase-3 and -9 activities

Caspase activity was detected using caspase-3 and -9 colorimetric assay kits (R&D Systems, Minneapolis, MN, USA). The assay procedures were followed according to the kit instructions. Protein concentration was determined using a Bio-Rad (Hercules, CA, USA) protein assay kit.

Statistical analysis

Paired data were evaluated by Student's *t*-test. A one-way ANOVA was used for multiple comparisons. A value of $P < 0.05$ was considered significant.

Fig. 2 Doxorubicin induces a dose- and time- dependent up-regulation of MITOL. (**A** and **C**) HL-1 cells were stimulated with the indicated doses of Doxorubicin (DOX), and harvested for immunoblotting. (**B**, **D** and **E**) HL-1 cells were stimulated with 5 μM Doxorubicin (DOX) and then harvested at the indicated time for immunoblotting. (**A** and **B**) It shows MITOL expression upon treatment with Dox. (**C** and **D**) It shows PARP cleavage upon treatment with DOX. (**E**) Immunoblots showing Bid, caspase-3 and Drp1 expression and cleavage upon treatment of cells with Dox. β-tubulin and β-actin served as a loading control. The densitometry data were expressed as the mean ± S.E.M. of three independent experiments. The relative expression level of protein was determined by dividing the percent value of specific protein to that of standard. *$P < 0.05$ *versus* non-treated control.
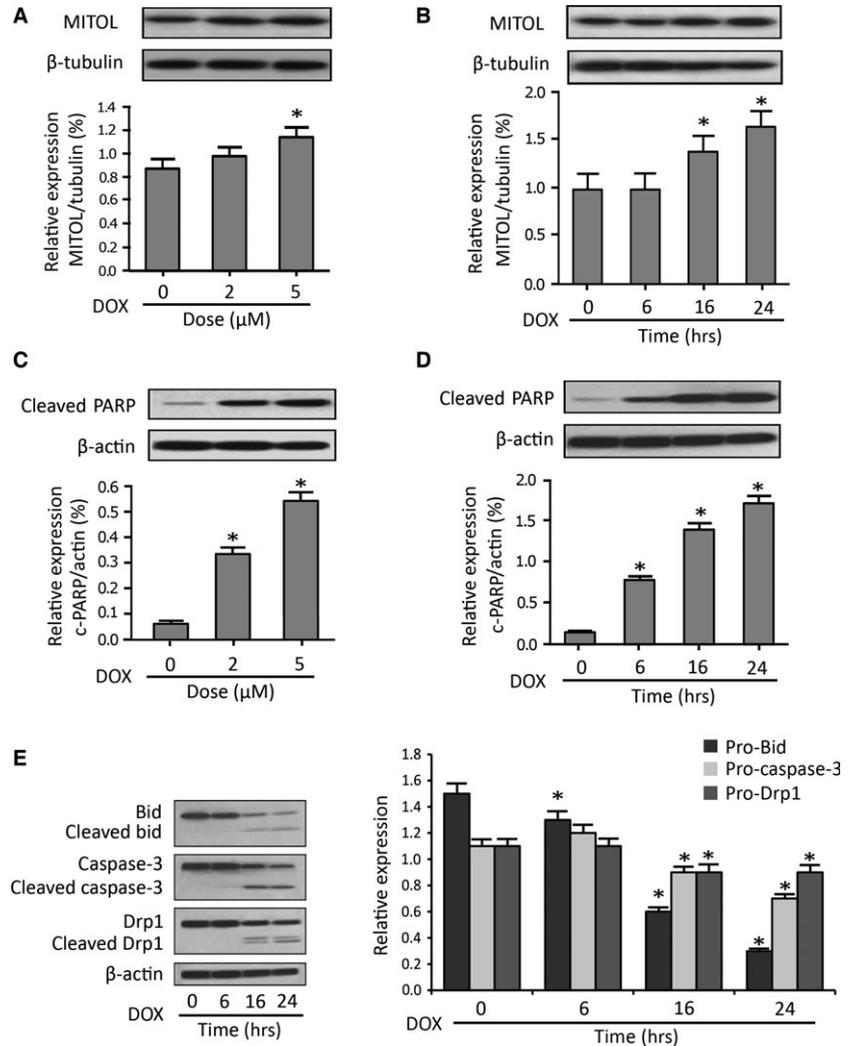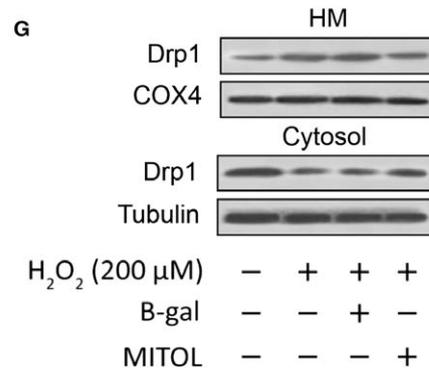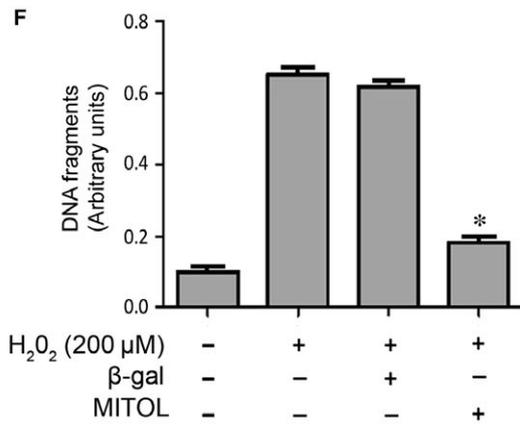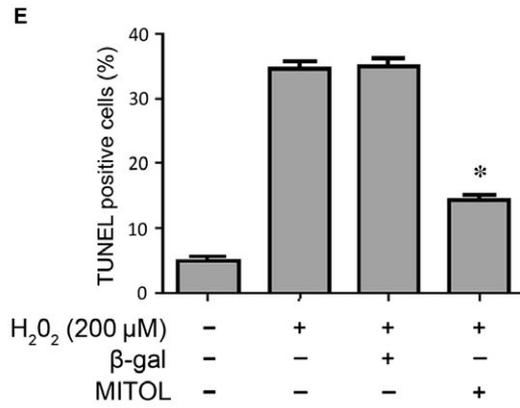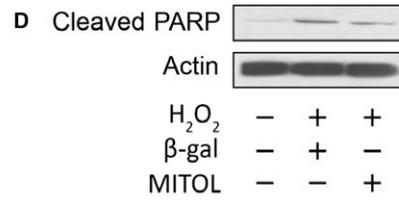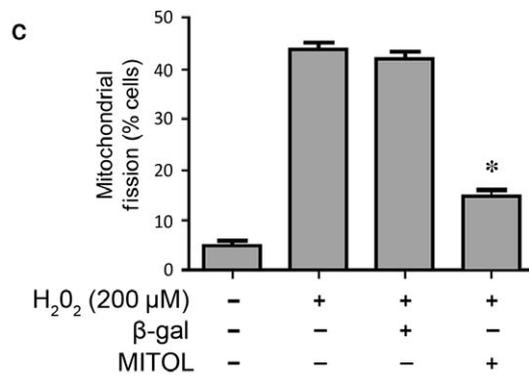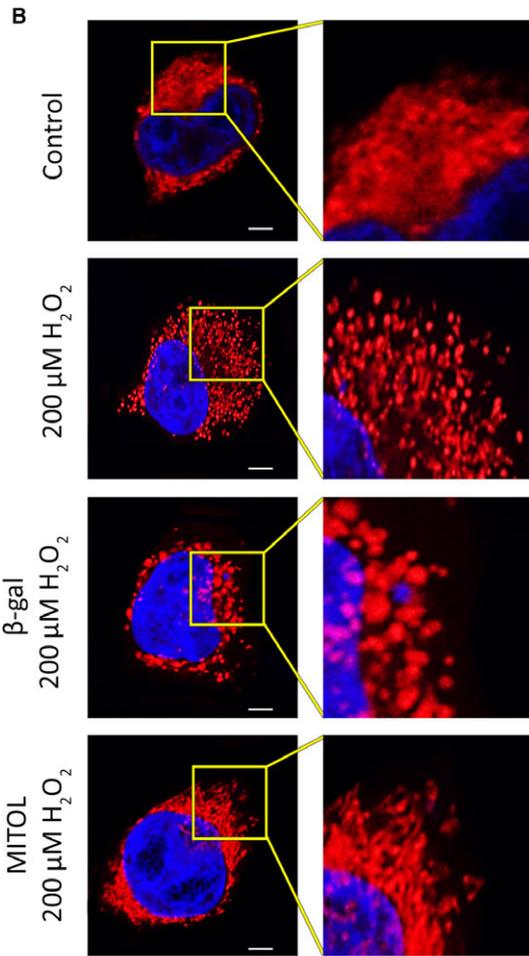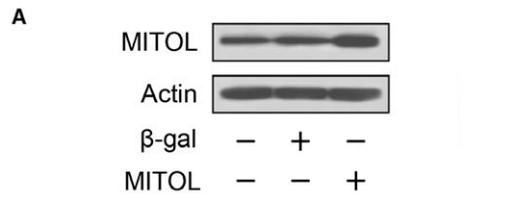
Results

MITOL is up-regulated in cardiomocytes upon apoptosis induction by H_2O_2 and doxorubicin

To test whether MITOL participates in the regulation of mitochondrial fission in cardiomyocytes, we treated cardiomyocytes with H_2O_2 or Dox to induce apoptosis and mitochondrial fission. We found that the expression levels of MITOL in mitochondria were increased upon H_2O_2 exposure (Fig. 1A and B). Meanwhile, PAPR and caspase-3 cleavage also increased in a same manner to H_2O_2 exposure (Fig. 1A and B), suggesting that MITOL could be a component in the apoptosis cascades induced by H_2O_2. Similarly, the expression levels of MITOL were increased upon Dox treatment in a dose- and time-dependent manner (Fig. 2A, and B). Concomitantly, we observed an increase in the cleaved-PARP (Fig. 2C, and D), suggesting that apoptosis occurred upon treatment with Dox. To further confirm the occurrence of apoptosis, we analysed for the levels of expression of other apoptotic factors. Our results demonstrated that both caspase-3 and Bid were cleaved. Strikingly, we observed a significant increase in the cleavage of Drp1 after Dox treatment (Fig. 2E). Thus, it appears that alteration in the levels of expression of MITOL is also associated with Dox-induced apoptosis in cardiomyocytes.

Overexpression of MITOL prevents mitochondrial fission and apoptosis

To determine if MITOL plays a critical role in mitochondrial fission, we expressed exogenous MITOL in cardiomyocytes. Transfection of cardiomyocytes with a MITOL expression vector resulted in elevated levels of MITOL expression (Fig. 3A and Fig. S1A, upper panel). Treatment with 200 μM of H_2O_2 caused mitochondrial fission; however, overexpression of MITOL inhibited mitochondrial fission as revealed by the mitochondrial morphology (Fig. 3B). Concomitantly, overexpression of MITOL could reduce the percentage of cells with mitochondrial fission (Fig. 3C). These data suggested that MITOL could prevent mitochondrial fission.

To determine if exogenous MITOL expression affected apoptosis, we analysed several apoptotic events. Our results showed that PARP cleavage was reduced (Fig. 3D and Fig. S1A, lower panel). We quantitatively analysed apoptosis by employing TUNEL staining and cell death ELISA, both of which specifically detect apoptosis. Mitochondrial ubiquitin ligase could attenuate apoptosis as indicated by reduced TUNEL staining (Fig. 3E) and DNA fragmentation (Fig. 3F).

These data suggested that exogenous MITOL is able to inhibit apoptosis in cardiomyocytes.

Finally, we detected the distribution of Drp1 and found that Drp1 accumulation in mitochondria was attenuated by MITOL (Fig. 3G and Fig. S1B). Thus, it suggests that exogenous MITOL could prevent Drp1 translocation.

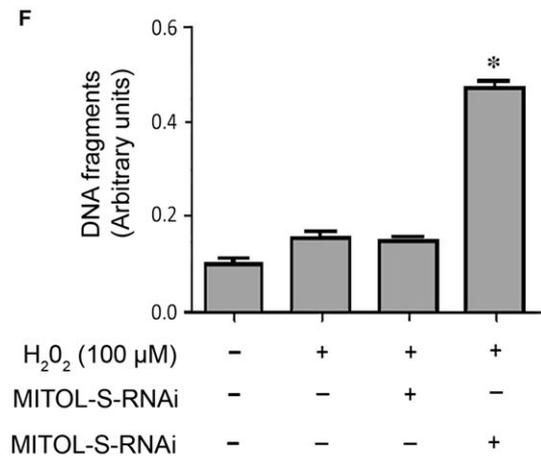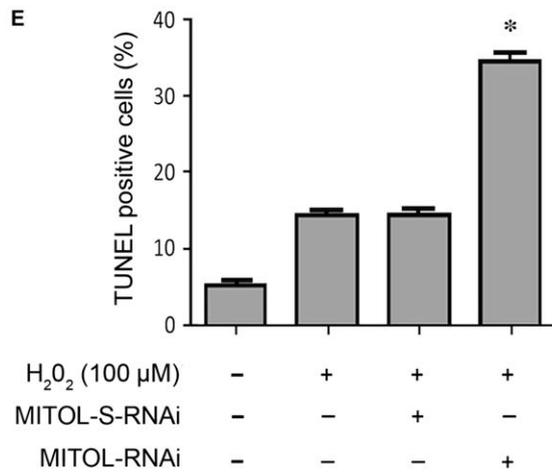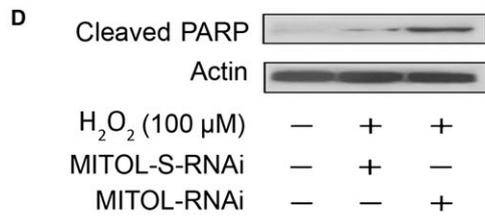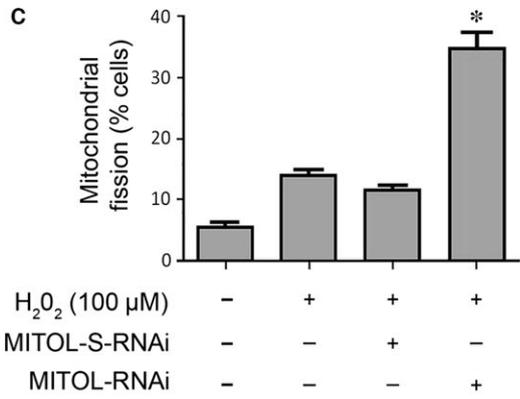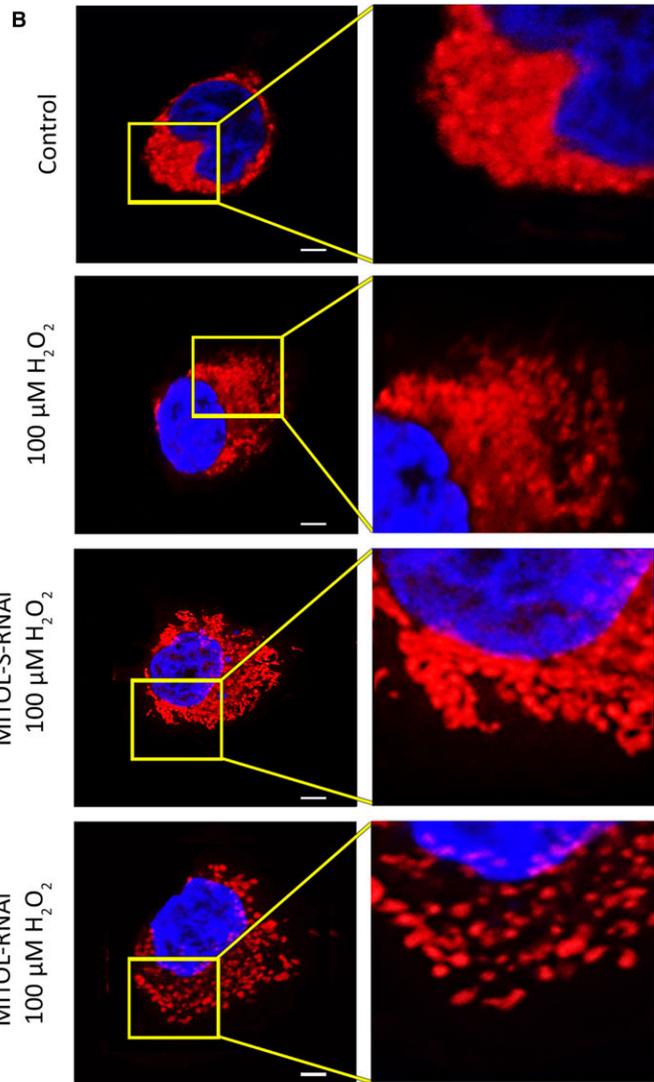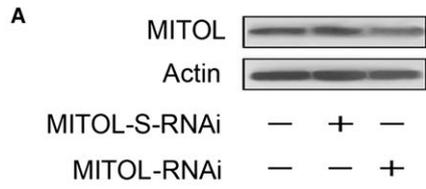## Knockdown of MITOL sensitizes cardiomyocytes to mitochondrial fission and apoptosis

To further investigate the role of MITOL in the regulation of mitochondrial fission, we detected the cell fate upon knockdown of MITOL. To this end, we produced and used an RNAi construct to knock down MITOL. As shown in Figure 4A and Figure S2, the siRNA could reduce MITOL expression. In cells expressing MITOL, exposure to H_2O_2 (at a low dose) led to no significant alterations in mitochondrial morphology; however, the same low dose of H_2O_2 caused mitochondrial fission upon knockdown of MITOL (Fig. 4B). Consistently, upon knockdown of MITOL, low dose of H_2O_2 caused a significant increase in the number of cells undergoing mitochondrial fission (Fig. 4C). Moreover, cleaved-PARP (Fig. 4D and Fig. S2), the percentages of TUNEL positive cells (Fig. 4E) and DNA fragments were significantly elevated (Fig. 4F), indicating an increase in apoptosis. These data suggested that endogenous MITOL participates in the inhibition of mitochondrial fission and apoptosis.

We further confirmed the effect of MITOL knockdown in primary neonatal rat cardiac myocytes. When MITOL-shRNA was used to knockdown endogenous MITOL, a higher percentage of cells underwent mitochondrial fission upon H_2O_2 exposure, compared to negative control and scramble shRNA-treated groups (Fig. 5A and B). Concomitantly, cell death ELISA showed that knockdown of MITOL increased H_2O_2-induced cardiomyocyte apoptosis (Fig. 5C). Therefore, these findings further support the anti-apoptotic effect of MITOL in primary cardiomyocytes.

MITOL prevents caspase-3 and caspase-9 activation

Apoptosis is executed by activated caspases, we therefore analysed for the activities of caspase-3 and caspase-9, two caspases that can be activated by mitochondrial apoptotic pathway. As shown in

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Fig. 3 Enforced expression of exogenous MITOL prevents mitochondrial fission and apoptosis. (**A**) Analysis of MITOL expression. Immunoblots show overexpression of MITOL in HL-1 cells. (**B** and **C**) Enforced expression of exogenous MITOL inhibits mitochondrial fission induced by hydrogen peroxide (H_2O_2). HL-1 cells were treated with H_2O_2. (**B**) It shows mitochondrial morphology, bar = 2 μm. Cells with mitochondrial fission (**C**). *$P < 0.05$ *versus* H_2O_2 alone. (**D**) Enforced expression of MITOL inhibits PARP cleavage. Cleaved-PARP was analysed by immunoblotting. (**E**) Enforced expression of MITOL attenuates apoptosis. HL-1 cells were treated with H_2O_2. Apoptosis was analysed by TUNEL assay. *$P < 0.05$ *versus* H_2O_2 alone. Data are expressed as the mean $\pm$ S.E.M. of three independent experiments. (**F**) Enforced expression of exogenous MITOL attenuates DNA fragmentation. HL-1 cells with and without exogenous MITOL expression were treated with H_2O_2. DNA fragments were analysed using the cell death detection ELISA. *$P < 0.05$ *versus* H_2O_2 alone. (**G**) Enforced expression of MITOL inhibits Drp1 accumulation in mitochondria. HM = mitochondria-enriched heavy membranes.

Fig. 4 Knockdown of MITOL sensitizes cardiomyocytes to mitochondrial fission and apoptosis. (**A**) Immunoblot shows MITOL knockdown in HL-1 cells. (**B** and **C**) Knockdown of MITOL sensitizes cells to undergo mitochondrial fission. HL-1 cells were exposed to H_2O_2. (**B**) It shows mitochondrial morphology, bar = 2 μm. (**C**) It shows cells with mitochondrial fission; *$P < 0.05$ *versus* H_2O_2 alone. (**D**) Immunoblot shows cleaved-PARP. (**E**) Knockdown of MITOL sensitizes cells to undergo apoptosis. Apoptosis was analysed by TUNEL assay. *$P < 0.05$ *versus* H_2O_2 alone. (**F**) Knockdown of MITOL sensitizes cells to undergo DNA fragmentation. DNA fragments were analysed using the cell death detection ELISA. *$P < 0.05$ *versus* H_2O_2 alone. Data were expressed as the mean ± S.E.M. of three independent experiments.
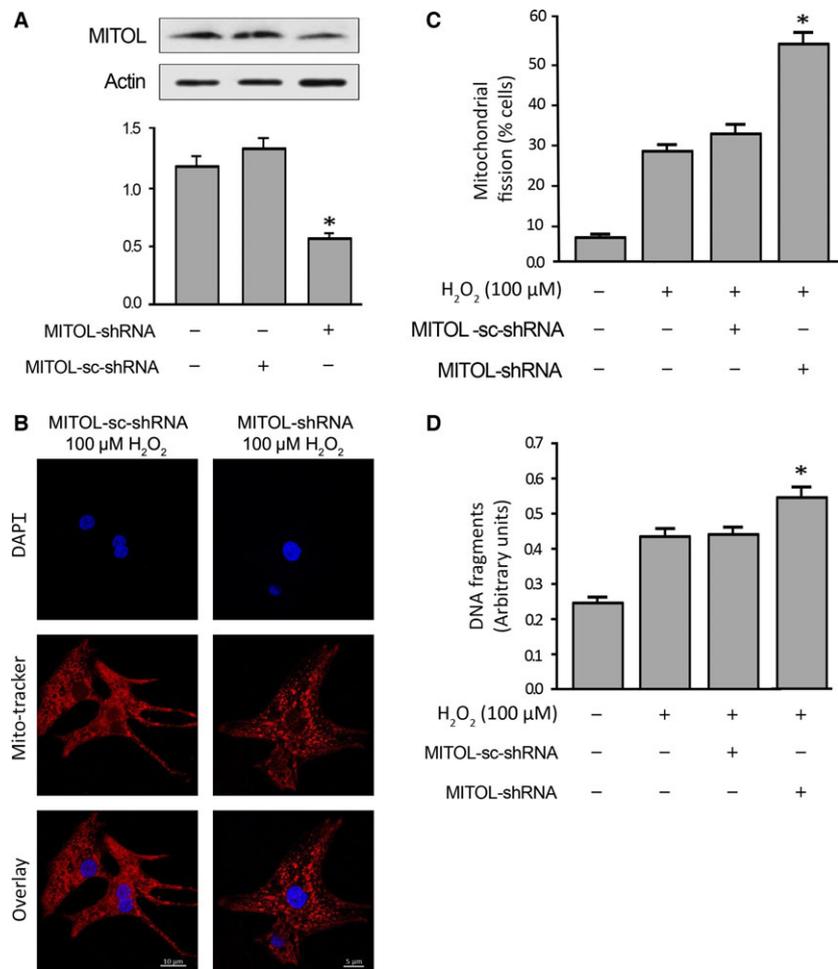
Figure 6A and B, H_2O_2 could activate caspase-3 and caspase-9, respectively, and enforced expression of exogenous MITOL led to a reduction in the activities of these caspases. Furthermore, inhibition of caspase-3 and caspase-9 by their inhibitors could attenuate apoptosis (Fig. 6C), suggesting that they played a critical role in cardiomyocyte apoptosis. These findings further support that MITOL regulates the Drp1-related mitochondrial fission and its downstream apoptotic pathway.
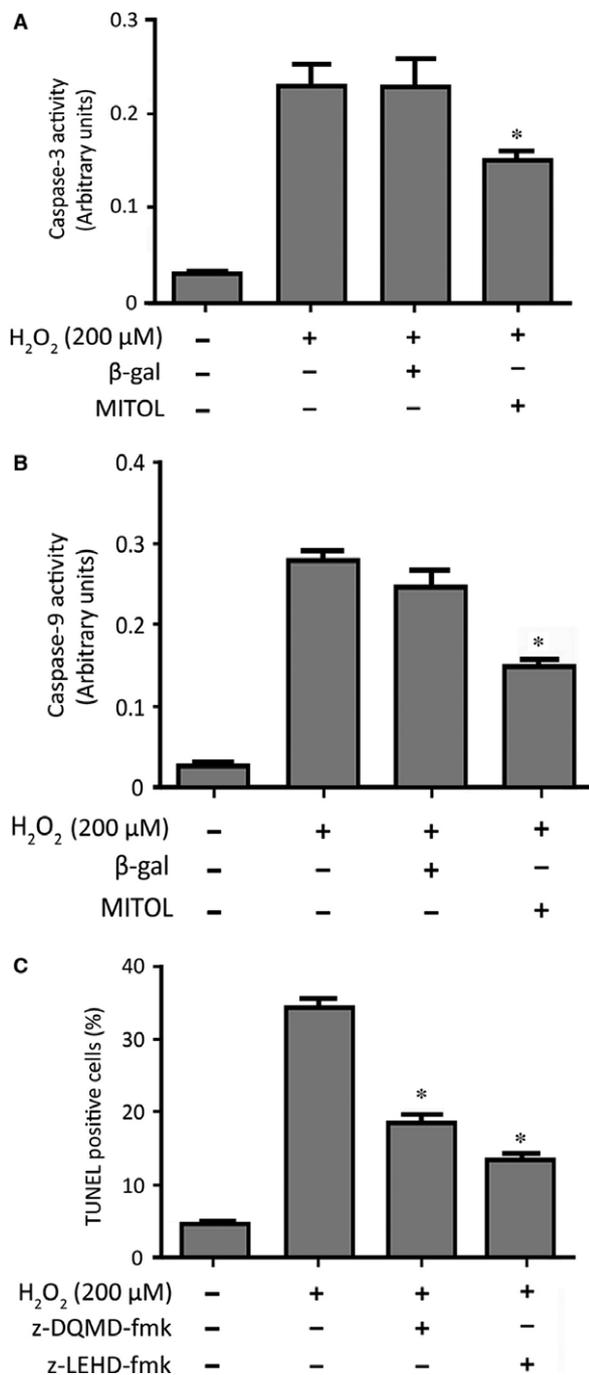
Discussion

Complex molecular mechanisms regulate apoptosis in cardiomyocytes [32]; and participate in the regulation of a variety of cardiac diseases [3–5, 33, 34]. Amongst those mechanisms is mitochondrial fusion and fission events, which controls apoptosis [10]. Mitochondrial ubiquitin ligase has been shown to be involved in mitochondrial dynamics and mitochondrial quality control [21, 23, 35, 36]. However, the exact role of MITOL in cardiomyocyte mitochondrial function is still obscure. In this study, we found that MITOL attenuates mitochondrial fission and apoptosis induced by H_2O_2 or Dox, most likely by inhibiting Drp1 accumulation in mitochondria. Mitochondrial ubiquitin ligase, an ubiquitin ligase, is characterized by its four transmembrane domains that help anchor the protein in the mitochondrial outer membrane [23–25]. Mitochondrial ubiquitin ligase interacts with both fission proteins (hFis1 and Drp1) as well as fusion proteins [23, 24, 37]. Mitochondrial ubiquitin ligase plays a critical role in regulating mitochondrial dynamic by regulating the function of mitochondrial

Fig. 5 Knockdown of MITOL increases hydrogen peroxide-induced mitochondrial fission and apoptosis in primary cardiomyocytes. (**A**, upper panel) Immunoblot shows MITOL knockdown in primary cardiomyocytes; (lower panel) the densitometry data were expressed as the mean ± S.E.M. of three independent experiments. The relative expression level of protein was determined by dividing the percent value of specific protein to that of standard. *$P < 0.05$ *versus* negative and scramble controls. (**B** and **C**) Knockdown of MITOL promotes mitochondrial fission. (**B**) It shows mitochondrial morphology. (**C**) It shows cells with mitochondrial fission; *$P < 0.05$ *versus* H_2O_2 alone. (**D**) Knockdown of MITOL promotes apoptosis. Apoptosis-related DNA fragmentation was analysed using the cell death detection ELISA. *$P < 0.05$ *versus* H_2O_2 alone. Data were expressed as the mean ± S.E.M. of three independent experiments.

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Fig. 6 MITOL inhibits caspase-3 and caspase-9 activation. (**A** and **B**) It shows Caspase-3 and Caspase-9 activity, respectively; *$P < 0.05$ *versus* H_2O_2 alone. (**C**) Inhibition of caspase-3 or caspase-9 attenuates apoptosis. HL-1 cells were treated with the inhibitor of caspase-3 (Z-DQMD-FMK) or caspase-9 (Z-LEHD-FMK) at 100 μM and then exposed to 200 μM of H_2O_2. Apoptosis was analysed by TUNEL assay. *$P < 0.05$ *versus* H_2O_2 alone. Data were expressed as the mean ± S.E.M. of three independent experiments.

fission and fusion proteins [21–23]. Dynamin-related protein 1 is one of the key proteins in the control of mitochondrial fission [13, 29, 38] and is involved in cytochrome c release from mitochondria into the cytosol [29]. Cytochrome c binds to apoptosis protease-activating factor 1 and procaspase-9 to form apoptosomes resulting in the activation of caspase-9 [39, 40].

In the current study, we found that MITOL overexpression inhibited Drp1 accumulation, and attenuated mitochondrial fission and apoptosis. Conversely, MITOL knockdown induced the cells to undergo mitochondrial fission and apoptosis. It has been noted that the MITOL-dependent Drp1 regulation tends to vary by cell conditions including cell cycle phase or nutritional status [23]. For instance, MITOL knockdown in mouse embryonic fibroblasts showed similar regulatory effect as has been shown in our current study [23]. But in human HeLa cells, a completely opposite effect was noted in that MITOL was required for Drp1-dependent mitochondrial division, and inhibition of MITOL leads to mitochondrial fusion in HeLa cells [24, 37]. Furthermore MITOL is required for degradation of mitochondrial fusion protein Mfn1 in LNCaP prostate cancer cells treated with CGP37157 (CGP), an inhibitor of mitochondrial calcium efflux, and resulted in mitochondrial fission. Comparably, knockdown of MITOL reduced Mfn1 degradation, which in turn elevated Mfn1 levels and thus, promoted mitochondrial fusion [41]. Another interesting study, using an *in-vitro* neuronal cell (RGC5) exposed to glaucoma-relevant stress conditions, found that mitochondrial fission was significantly blocked in cell expressing inactive MITOL and did significantly delay the cell death [42]. These evidence further substantiate the contrasting function of MITOL in different cell types under various cellular stress conditions.

The specific roles of MITOL in mitochondria have not yet been fully elucidated. It is reported that MITOL is able to eliminate misfolded proteins localized in mitochondria, such as mutant superoxide dismutase 1 [43], and mutant short chain acyl CoA dehydrogenase [44], which exacerbate neuronal disorders. Thus, MITOL plays an important role in protecting neuronal cell death by degrading the accumulated denatured proteins in mitochondria [23]. It is of note that mitochondrial fission and fusion are controlled by a complex molecular mechanism in which a variety of proteins such as optic atrophy 1, hFis1, and mitochondrial division 1 are involved [14, 16, 45]. It is critical for future studies to investigate whether these molecules participate in the regulation of mitochondrial fission process in the heart. Therefore, it is also necessary to elucidate whether ubiquitination by MITOL is required for the regulation of mitochondrial dynamic. Interestingly, we noticed that mitochondrial morphology in HL-1 cell lines in fusion state were packed into a network rather than long thin filamentous configuration, which is commonly seen in cardiac fibroblasts (Fig. S3). This morphological discrepancy highlights that mitochondrial configuration in different cell types can be varied depending on their functions and histological backgrounds [10, 30, 46, 47].

In conclusion, this study reveals that MITOL is involved in the mitochondrial fission machinery of cardiomyocyte apoptosis. Mitochondrial ubiquitin ligase attenuates the mitochondrial fission induced by H_2O_2 and blocks Drp1 accumulation in the mitochondria of

cardiomyocytes. Thus, MITOL may serve as a novel therapeutic target for apoptosis-related cardiac diseases.

Acknowledgements

Conflict of interest

The authors confirm that there are no conflicts of interest.

Author contribution

Designed the experiments: PL, JW and LHHA. Performed the experiments: LHHA and JW. Analysed the data: LHHA, JW and PL. Wrote the paper: LHHA, JW, PL and BP.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 (**A**, upper panel) Quantitative densitometry of immunoblot for expression levels of MITOL (corresponded to Fig. 3A, *P* < 0.05 *versus* negative control or β-gal) and (lower panel) PARP cleavage (corresponded to Fig. 3D, *P* < 0.05 *versus* negative control or β-gal treated with 200 μM H_2O_2).

Figure S2 (upper panel) Quantitative densitometry of immunoblot for expression levels of MITOL (corresponded to Fig. 4A, *P* < 0.05 *versus* MITOL-S-RNAi) and (lower panel) PARP cleavage (corresponded to Fig. 4D, *P* < 0.05 *versus* non-treated control or MITOL-S-RNAi treated with 100 μM H_2O_2).

Figure S3 Mitochondrial morphology during fusion state in non-treated rat primary cardiac fibroblast (**A**), rat primary cardiomyocyte (**B**) and HL-1 cells (**C**).

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